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Exploring effects of stress from a cellular and molecular perspective

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Chronic stress and long-term antidepressant administration: neuroendocrine and immunohistochemical changes in the female rat brain

"The greatest lesson in life is to know that
even fools are right sometimes"
Sir Winston Churchill

Antidepressant actions in the female brain

Whereas the previous two chapters provided experimental evidence to substantiate the negative consequences of stressful events on neuronal functioning in male and female rats, this section will focus on the neurochemical adaptations induced by long-term antidepressant treatments in cyclic female rats and their ability to correct stress-induced neuronal abnormalities. Three different classes of antidepressants were tested here including a serotonin reuptake enhancer (tianeptine), a selective serotonin reuptake inhibitor (citalopram), and a selective norepinephrine reuptake inhibitor (reboxetine). These compounds were chosen in consideration of their efficacy in the treatment of stress-related neuropsychiatric disorders, such as panic, anxiety, post-traumatic stress disorder and, more importantly, depression. The decision to combine prolonged stress exposure with simultaneous antidepressant administration stems from the fact that depressed subjects have a long history of stress both before and during pharmacotherapy while experimental animals used to test antidepressants are rarely exposed to concomitant stressful conditions. Clinical evidence suggests that adverse events do not only contribute to the development and/or maintenance of psychopathology in humans [1-3], but also seem to affect the 'therapeutic power" of antidepressants [4]. This investigation was limited to female rats with respect to the fact that most stress-related psychiatric illnesses are characterized by marked gender-related prevalence [5]. Although animal models have provided valuable information regarding possible mechanisms underlying the pathophysiology of these complex diseases [6,7] as well as antidepressants' modes of action [8,9], important discrepancies still exist between experimental models and human psychopathology. For instance, although women have a higher susceptibility to mood disorders, constituting the majority of patients receiving antidepressant treatment, most of the preclinical research has been performed in male animals [10]. In the present chapter, we thus explore the neurochemical changes induced by prolonged footshock exposure and/or concomitant long-term antidepressant treatments, in an attempt to answer a crucial question: are long-term antidepressant treatments similarly effective in preventing/reversing the neurohistochemical changes induced by chronic stress?

Stress and depression: is there a connection?

Depression is a heterogeneous disorder in which different etiological causes, including environmental factors (stressful life events) [3,11] and genetic factors ("vulnerability" or "predisposition") [12], interact in multiple and complex manners [13]. Clinical studies have confirmed the importance of adverse experiences in the development of psychopathology and a strong correlation between stressful life events (SLEs) and the precipitation of depression has been demonstrated, especially in women [3,14,15]. Kendler

and colleagues have speculated that the association between SLEs and major depression accounts for approximately 75% while genetic risk factors account for the remaining 25%[3]. Although it is difficult to attribute a numeric value to the role played by adverse experiences in the development of depression, the significance of this study is owed to the notion that environmental factors may overrule genetic influences and lead to depression independently of genetic vulnerability or predisposition [3,11].

The brain responds to aversive stimuli in a complex yet orchestrated manner. The loss of organization often seen in this response may play a crucial role in the occurrence of neuronal dysfunctions. Preclinical studies have suggested that stress exerts its deleterious influence on the brain by promoting long-term changes in multiple neurotransmitter systems and this action may increase the vulnerability to the development of psychiatric illnesses [16-18]. A leading hypothesis proposes that the impact of stressful events is greater in the initial stage than in the subsequent episode of major depression; the strength of the relationship progressively declines as the number of previous depressive episodes increases (a theory known as the "kindling hypothesis")[19,20,21]. SLEs have been found to be strongly associated with subsequent episodes of depression [3]. The depressogenic effects of adverse experiences seem thus to be concentrated in the period immediately subsequent to the occurrence of such events. Although environmental factors also play a key role in depression, several other investigations have underlined the importance of genetic influences. Two theoretical models that might explain the relationships between these two main risk factors are known as "additive" and "genetic control of sensitivity to the environment model". In the additive model, the increased risk associated with exposure to adverse conditions is similar for individuals with low-risk and high-risk genotypes. This model predicts that the depressogenic impact of SLEs and genetic factors is independent. In contrast, the second model proposes a greater risk of developing depression associated with SLE exposure for those with a high-risk genotype. Genes do influence the risk of depression by altering the individual's sensitivity to the depressogenic effect of SLEs [14]. Clinical evidence seems to support the latter model [15].

Another important aspect in the association between adverse experiences and depression regards the impact of multiple SLEs [22]. To explain this relationship, three plausible theoretical frameworks have been proposed. The most simple or additive model suggests that the impact of SLEs is independent of the occurrence of other events. Multiple SLEs might positively interact and their depressogenic effect increases when it co-occurs with others. The positively interactive model proposes a reservoir of "coping ability" that might withstand the impact of one event but could be overwhelmed by multiple episodes. The negatively interactive model, instead, introduces a threshold for stress that, if exceeded, has no additional impact on depressive risk. Clinical evidence focused on severe SLEs, tends to best support the negatively interactive model. After one

severe SLE, little or no increased risk for depression was found given additional SLEs. Kendler and colleagues, who examined multiple SLEs occurring together in the same month, support the "positively interactive model": the impact of increasing numbers of SLEs on risk for a depressive onset is significantly greater than predicted by an additive model [22].

The monoamine hypothesis of depression

The monoamine hypothesis was coined over 30 years ago [23,24] and suggests an underlying biological basis for depression, namely a deficiency of the monoamine neurotransmitters norepinephrine, serotonin and/or dopamine in the central nervous system [25]. Since the stimulation of the monoaminergic system has been associated with clinical improvement, various classes of antidepressants that act by increasing monoamine levels within the synaptic cleft, either by inhibition of their degradation or by blockade of their reuptake, have been developed [26]. During the past decade, selective serotonin reuptake inhibitors (SSRIs) have become established as the treatment of choice for affective disorders [27]. However, newer antidepressants that selectively elevate norepinephrine levels or act simultaneously on different neurotransmitter systems have also proven to effectively alleviate depressive symptom [28-30].

Although substantial evidence exists to support a role of monoamine systems in the mechanism of action of antidepressants, intensive investigation has failed to find conclusive affirmation of a primary dysfunction in specific monoaminergic systems in subjects with major depressive disorders [31-33]. Moreover, there are several major issues that have not been addressed by the monoamine hypothesis. These problems concern mainly the mode of action of antidepressants acting on serotonergic and noradrenergic systems and include:

- **Efficacy**: in clinical trials, antidepressants, especially the newest generations of drugs including SSRIs, Norepinephrine Reuptake Inhibitors (NARIs), and selective Serotonin/Norepinephrine Reuptake Inhibitors (sSNRI), appear to be effective in approximately 60% of the subjects suffering from depression [34]. Although the first tricyclic agent (TCA) was introduced more than 30 years ago, the newest SSRIs or NARIs have failed to demonstrate an enhanced efficacy compared to these older antidepressants [35,36]. Newest antidepressants however, are better tolerated and do not show the serious cognitive, cardiac, and somatic side effects that characterize long-term TCA treatment [37-44].
- **Selectivity**: it is clear that SSRIs, NARIs, and dual SNRIs act through the stimulation of serotonergic and noradrenergic systems. There is still some confusion however, regarding the specific cellular or molecular targets underlying their therapeutic action, which include neurotransmitter transporters, specific receptors, intracellular proteins, enzymes, and transcription factors [45-48]. Various lines of evidence indicate

that selectivity of these agents dissipates following long-term administration. After several weeks, even highly selective drugs such as SSRIs or NARIs, affect the activity of numerous neurotransmitter systems and brain structures, some of which are not directly linked with the pharmacological profile of the antidepressant [49-51]. An intriguing possibility is thus that this limited selectivity following long-term treatment, rather than their high specificity observed in pharmacological essays, might represent the critical factor for their beneficial effects [52].

- **Mode of action**: an additional question to be addressed concerns the molecular substrates involved in the modulation of antidepressants' therapeutic effects. In contrast to those medications acting through the potentiation of monoaminergic transmission (TCAs, SSRIs, NARIs, and dual SNRIs), other effective antidepressants exert their pharmacological action by enhancing serotonin reuptake (tianeptine) [53,54] or modulating the activity of selective enzymes and/or transcription factors that are not directly linked to monoamine metabolism or signaling transduction pathways (such as lithium and valproate) [55,56].
- **Delayed therapeutic action**: while side-effects are manifested within hours or days, the beneficial effects of antidepressants are delayed and can take several weeks or even months to appear, causing considerable problems with patient compliance [57-59].
- **Monoamine depletion studies**: experimental monoamine depletion exacerbates depressive symptoms only in depressed subjects successfully treated with SSRIs or NARIs. Monoamine depletion failed to induce the same negative effects in medication-free symptomatic patients or healthy subjects. This implies that serotonergic and/or noradrenergic dysfunctions are unlikely to be the primary cause of depression although they may play a critical role in the mechanisms by which antidepressants act [32,33,60,61].

These findings suggests that while the potentiation of monoaminergic neurotransmission is fundamental for the modulation of antidepressants' action, only fragmentary evidence supports a primary role for monoamine deficiencies in depression. Depression is a heterogeneous disease in which numerous factors are involved. Furthermore, the complex nature of this disorder may favor its occurrence in a multiplicity of different forms. Monoaminergic deficiencies may represent just a feature of the "depressive syndrome" and characterize only a limited number of subtypes of depression. Another possibility is that monoamine deficiencies may constitute one of the multiple consequences associated with the course of the disorder. Monoaminergic systems are extensively distributed within the brain [62-64] and it is not surprising that clinical research, throughout the years, has identified, in depressed subjects, abnormalities in the noradrenergic, dopaminergic, and cholinergic system as well as an impaired HPA axis regulation [65-70]. The behavioral and physiological manifestations of this psychiatric illness are complex and undoubtedly mediated by multiple networks of interconnected

neurotransmitter pathways. The abnormal activity of one or more key components may alter the coordinated regulation of an entire system, generating a "domino-like effect" that ultimately disrupts its ability to react to incoming stimuli with appropriate responses. Depression may be better viewed as a complex set of varying symptoms rather then an homogenous disorder, since it exhibits heterogeneous pathology with several different etiological causes yet few common consequences, such as disrupted cortical-limbic function, responsible for most of the deficits associated with the illness (cognitive impairment and emotional dysregulation) [70,71]. Revelations in the understanding of this psychiatric disorder and its treatment call for a clear comprehension of the factors and mechanisms leading to the above-mentioned functional and morphological abnormalities in cortical and subcortical structures.

Molecular and cellular theory of depression: the STRESS-BDNF hypothesis

Although the association between adverse experiences, brain abnormalities, and the occurrence of depression appears to be consistent, much less is known about the neurobiological substrates underlying stress-induced cortical-limbic defects. Stress deeply affects neuronal functional and structural integrity, inducing alterations at the cellular and the molecular level. Molecular changes include modifications of gene expression, protein synthesis and phosphorylation, while cellular changes include dendritic remodeling and/or atrophy, reduced neurogenesis, and possibly neuronal death [72-74]. Advances in molecular techniques have enhanced our insights into the mechanisms underlying the deleterious influence of stress on brain functions as well as the relationships between intracellular abnormalities and psychopathology. In the past few years, a growing number of studies have begun to characterize stress and antidepressant action beyond neurotransmitter and receptor level. This work has demonstrated that multiple intracellular pathways are involved in the transduction and modulation of antidepressant effects [45,75]. Despite the complexity of the intracellular apparatus, growing evidence suggests that the final result of antidepressant action may involve the stimulation of a limited number of "common effectors". One such final mediator, which appears to be a common molecular target of several classes of antidepressants affecting both serotonergic and noradrenergic neurotransmitter systems, is CREB [45,76-78]. CREB regulates the transcription of specific genes, including those codifying for BDNF and TrkB receptor [79-81]. Stress may precipitate depression through its detrimental action on neuronal plasticity achieved by limiting BDNF synthesis and release. Interestingly, experimental data points in the direction of a chronic stress-induced inhibition of CREB phosphorylation and/or BDNF expression. This stress-mediated inhibition may thus provide a theoretical mechanism through which sustained

stress exposure may reduce neuronal plasticity and, ultimately, lead to selective cortical-limbic abnormalities.

Remodeling of synaptic contacts, growth and branching of dendrites are only a few examples of neuronal plasticity. This dynamic process is based on the ability of neuronal systems, brain structures, single neurons, synapses and receptors, to adapt to alterations in the internal and/or external environment by modifying specific structure and functions [82]. To support these dynamic changes, new neurons are also produced in the hippocampus. Neurogenesis has been reported in rats, tree shrews, macaques, and humans, demonstrating that adult-generated neurons are a common feature of the mammalian brain [83,84]. Neurogenesis and neuronal plasticity however, are affected by stress [85,86]. Prolonged stress disrupts dendrite growth and branching [17], causing atrophy[87] and, in severe cases, neuronal death [74,88]. Acute and chronic stress have been shown to suppress neurogenesis, especially in the adult brain [83]. It is important to note that these forms of neuronal plasticity are crucial for proper functioning of the brain and numerous psychiatric disorders are characterized by reduced hippocampal neurogenesis and neuronal atrophy [73,84,89-92]. Reduced hippocampal activity and volume have also been observed in depressed subjects [93,94]. Therefore, although affective disorders have traditionally been conceptualized as neurochemical disorders, there is now considerable literature demonstrating that these illnesses are also associated with significant reductions in regional central nervous system (CNS) volume and cell numbers. Structural changes observed in depression however do not appear to be limited to the hippocampus. Several recent postmortem studies have also documented prefrontocortical abnormalities [95-97], including reductions in the number and density of cortical neurons and glial cells [98]. In the prefrontal cortex, a histological study of area sg24 located in the subgenual prefrontal cortex found striking reductions in glial cell number in patients with familial major depression (24% reduction) and manic-depressive illness (41% reduction), as compared with healthy subjects [95]. Together, these findings provide convincing evidence that decreased regional CNS volume, due to reduction in cell numbers, dendritic atrophy, and/or inhibition of neurogenesis, may lead to cortical-limbic impairments that, ultimately, promote psychopathology.

Neurotrophins participate in a broad range of functions including synaptogenesis, growth, differentiation, and survival [99-101]. Neurotrophic factors such as BDNF have also been shown to enhance the length and complexity of dendritic trees in cortical neurons [102,103]. These crucial activities require the coordinated interactions between multiple mediators, including receptors (Trk receptors), enzymes (PI3K and ERK1/2) and transcription factors (CREB) [104-109]. Activation of Trk receptors following the binding of specific ligands triggers a complex sequence of intracellular events that begins with receptor autophosphorylation, is followed by the activation of several downstream signaling cascades, and culminates with the stimulation and/or inhibition

of the expression of selective genes [107,108,110]. New insights into the role of neurotrophin signaling pathways in the pathophysiology and treatment of depression have been provided by the large number of studies reporting alterations in the expression of one or more members of these cascades in depressed subjects before as well as after antidepressant treatment [76,78,111-116]. Neurotrophin expression as well as intracellular cascades involved in the transduction of trophic signals thus appear to represent common targets of antidepressant action, independent of their pharmacological profile:

- **Serotonin and/or norepinephrine re-uptake inhibitors**. cAMP-mediated regulation of gene transcription has been implicated in the activity of numerous antidepressants acting on serotonin and/or norepinephrine neurotransmitter systems [45]. It has been proposed that CREB might represent the main effector in the modulation of antidepressants' beneficial action [76,78,115]. Chronic SSRI/NARI administration increases CRE-mediated gene expression and CREB phosphorylation in a region- and drug-specific manner [117,118]. The most consistent effects have been observed in the amygdala, hippocampus, and prefrontal cortex [119]. More importantly, induction of CRE-mediated gene expression and CREB phosphorylation were not observed in response to acute pharmacological treatment, which is consistent with the time course of therapeutic action of these drugs [117]. Antidepressant-induced CREB phosphorylation has been reported, as mentioned above, in selective subcortical regions, such as the amygdala [119], and numerous reports have confirmed the importance of this structure in the modulation of some of the behavioral actions of antidepressants [119]. The amygdala also modulates fear-related responses and conditioned avoidance behaviors [120,121]. The possibility that changes in CREB expression and/or phosphorylation may influence the function of this subcortical area is also supported by recent observations, which illustrate that overexpression of CREB in the amygdala alters fear-related memory formation [122,123]. It is also plausible that chronic stress-induced neurochemical changes in the amygdala could promote abnormal cognitive and emotional processing, often observed in depressed subjects, and that long-term antidepressant treatments may correct these alterations [117,119].

  In addition to CREB, another downstream target of both SSRIs and NARIs is BDNF. BDNF expression has been found reduced following stress [124-126] and in depression[114]. Relevance of this neurotrophin in the regulation of neuronal functions has led to the hypothesis that its reduced availability may constitute a critical predisposing factor for the development of neuronal defects and, ultimately, psychopathology [127]. This view is also strongly supported by the antidepressant-like effects of BDNF [128,129] and by the ability of long-term antidepressant treatment to enhance its expression [117,130]. The possibility that antidepressant-induced stimulation of BDNF expression involves CREB is supported by the presence of CRE in the

promoter of BDNF gene [81]. Enhanced CREB expression and phosphorylation induced by long-term antidepressant treatment may ultimately reverse stress-induced reduction of BDNF expression, thereby preventing the deleterious consequences associated with limited availability of this neurotrophin on hippocampal and cortical neurons. A vital role for CREB and BDNF in the pathophysiology of depression and antidepressants' beneficial action has also been suggested by a recent post-mortem investigation documenting reduced CREB and BDNF expression in depressed subjects [112]. More importantly, elevation of cortical CREB levels was found in patients receiving antidepressant medications prior to death [45].

- **Serotonin re-uptake enhancer: tianeptine.** Tianeptine is an atypical antidepressant agent, both in terms of structure (modified tricyclic agent) and pharmacodynamic profile. This antidepressant, unlike traditional TCAs and SSRIs, stimulates the reuptake of serotonin [131]. Despite its alternative neurochemical profile however, tianeptine is effective in the treatment of both major depression and bipolar disorder, with a clinical efficacy similar to TCAs or SSRIs [53,132].

  The human hippocampus undergoes atrophy in the aftermath of severe stress, recurrent depression, and Cushing's syndrome [89,133-135]. Prolonged psychological stress is also associated with loss of hippocampal neurons in monkeys [136] and with dendritic atrophy in the hippocampal CA3 region in both rodens and primates [137,138]. This atrophy affects apical dendritic trees, comprises a reduction in length and branching, and seems to be the result of alterations of dendritic cytoskeleton [139]. Three main factors mediate hippocampal damage, including endogenous excitatory amino acids, serotonin and glucocorticoids [140,141]. Massive serotonin release occurs in response to stress [142-144]. In addition, stress stimulates the release of excitatory amino acids from mossy fiber synapses and steroid hormones from adrenal glands. These events are not harmful on their own but only when they occur concurrently, since the synergy of their molecular and cellular actions augments their negative effects. Long-term tianeptine administration has been reported to prevent stress-induced atrophy of CA3 pyramidal neurons whereas neither fluoxetine nor desipramine has such effects [54,145]. Tianeptine treatment was also effective in preventing stress-induced learning impairments [54]. Molecular mechanisms underlying the positive action of this atypical antidepressant in preventing stress-mediated hippocampal abnormalities are still a matter of debate however. Several reports have shown the ability of tianeptine to correct stress-induced disturbances of the stress response[146,147]. Hyperactivity of the HPA axis, a common abnormality associated with sustained stress exposure and depression [148,149], may lead to permanently elevated glucocorticoid concentrations. Stress-induced elevation of glucocorticoid level together with massive release of serotonin and glutamate may thus create the

conditions for the development of neuronal defects. Remarkably, the pharmacological profile of tianeptine makes this drug the ideal candidate for the prevention of these abnormalities, since:

1. the serotonin-enhancing nature of this antidepressant allows active removal of serotonin from the synaptic cleft [150];
2. tianeptine has been reported to be effective in preventing stress-induced HPA axis hyperactivity [146,147], which may limit brain exposure to high glucocorticoid levels;
3. recent reports have also presented evidence concerning tianeptine's ability to interfere with glutamatergic transmission [151].

Tianeptine thus seems to exert its actions at multiple levels. It is tempting to speculate that the prevention of interactions between serotonin, glutamate, and glucocorticoids, following the exposure to stressful events, accounts for this antidepressant's ability to limit their harmful synergy, thereby preventing subsequent development of neuronal abnormalities.

Materials and Methods

Animals

Cyclic female Wistar rats were used in this investigation (n° = 25: 195-212 g). The animals were individually housed (cages 45 x 28 x 20 cm) with food and water available ad libitum. They were maintained on a 12/12-hr light/dark cycle, weighed (9:00 hr) and handled daily for 5-8 min to minimize non-specific stress response. The experiments were carried out in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC), and with the guidelines of the Animal Bio-ethics Committee of the University of Groningen (FDC: 2509).

Chronic footshock procedure

The rodent footshock-chamber consists of a box containing an animal space placed on a gridfloor connected to a shock generator and scrambler. Stressed rats received one session of 30-120 min/day in the footshock box during which 5 inescapable footshocks were applied (0.8 mA in intensity and 8 sec in duration) with different inter-shock intervals in order to make the procedure as unpredictable as possible.

Experimental Setup

To investigate the immunohistochemical alterations induced by sustained stress exposure, long-term antidepressant treatments, as well as to examine the interactions between experimental conditions, a 2 X 2 procedure was applied. Rats were randomly assigned to eight groups:

1. **CTR-vehicle** (n° = 6): these rats were exposed, daily, to the footshock box and received vehicle injections. However, these animals were not subjected to footshocks during the experiment;
2. **STRESS-vehicle** (n° = 6): stressed females were exposed, daily, to the footshock procedure (5 electric footshock during an interval of 30-120 minutes) and received vehicle injections;
3. **CTR-tianeptine** (n° = 6): these animals were used as controls to identified neurochemical adaptations induced by long-term tianeptine administration. For this reason, these rats were exposed, daily, to the footshock box and received tianeptine injections. However, they were never exposed to footshocks during the experiment;
4. **STR-tianeptine** (n° = 7): these females were exposed, daily, to the footshock procedure and received tianeptine injections;
5. **CTR-citalopram** (n° = 6): these animals were exposed, daily, to the footshock box and injected with citalopram. They were never subjected to footshocks;
6. **STRESS-citalopram** (n° = 7): stressed females were exposed, daily, to the aversive procedure and received citalopram injections;

7. **CTR-reboxetine** (n° = 6): these rats were exposed, daily, to the footshock box and injected with reboxetine. They did not receive footshocks throughout the experiment.
8. **STR-reboxetine** (n° = 7): these animals were exposed, daily, to the footshock procedure and injected with reboxetine.

On the final day of the experiment all rats were placed in the footshock box. However, during this final session, of the duration of 15 minutes, no footshocks were delivered. It is also important to note that no injections were administered prior of this final session allowing 24-hour washout period.

Pharmacological profile, mode of action, dosage, and route of administration.

Citalopram. The mechanism of action of this SSRI is presumed to be linked to the potentiation of serotonergic activity in the CNS resulting from inhibition of neuronal reuptake of serotonin (5 HT) [152,153]. Citalopram is a racemic mixture (50/50) and the inhibition of serotonin reuptake is primarily due to the (S)-enantiomer. *In vitro* and *in vivo* animal studies suggest that this compound is a highly selective SSRI with minimal effects on norepinephrine and dopamine neuronal reuptake [152,154]. Moreover, this antidepressant has no or very low affinity for 5-$HT_{1A,}$ 5-HT_{2A}, dopamine D_1 and D_2, apha$_1$-, alpha$_2$-, and beta-adrenergic, histamine H_1, gamma aminobutyric acid (GABA), muscarinic cholinergic, and benzodiazepine receptors [154]. Antagonism of muscarinic, histaminergic, and adrenergic receptors has been hypothesized to be associated with various anticholinergic, sedative, and cardiovascular effects of other psychotropic drugs.

Dosage. Citalopram, kindly provided by Lundbeck B.V. (The Netherlands), was dissolved in saline (0.9% NaCl) at a concentration of 20mg*ml^{-1}, and injected intraperitoneously (i.p.) at the dosage of 20mg*kg^{-1}*day^{-1}, for a 21 day-period, 30-45 minutes before exposure to the footshock procedure. The daily dose of 20mg*kg^{-1}*day^{-1} was chosen after reviewing recent pharmacological studies using this antidepressant in a long-term setting [155,156]. These studies suggested that we could rely on the administered dosage of 20mg*kg^{-1} to provide a sufficient plasma concentration of about 250-300 nmol*l^{-1} [156,157], independent of the way of administration (oral vs. osmotic pumps). A plasma concentration of 250-450 nmol*l^{-1} was also observed after chronic citalopram administration through diet (10 and 40 mg*kg^{-1} daily) or oral administration (40 mg*kg^{-1} daily). It is noteworthy that serum citalopram concentrations around 100 nmol*l^{-1} were observed in humans receiving repeated oral doses within a normal dose range [158]. We can thus safely assume that plasma concentrations at least as high as those reported in clinical practice were also reached in the present study using a daily dosage of 20 mg*kg^{-1} administered through i.p. injections for a three-week period. Interestingly, as reported by Kugelberg and colleagues (2001), citalopram levels were constantly higher in the brain compared to those observed in the serum independent of the dosage administered [156]. The ratios between citalopram concentration in the serum and in the brain were constant for each drug concentration administered (10, 20 and 100 mg*kg^{-1}). Furthermore, the

antidepressant levels were 1.5-2 times higher in the cerebral cortex compared to the levels found in the mesencephalon-pons [156].

Tianeptine. Tianeptine is an atypical antidepressant, both structurally and in terms of its neurochemical profile. It is devoid of sedative effects and induces slight stimulation of locomotor activity. In monkeys, it decreases aggressive and emotive states and improves individual behavior [159]. Pharmacological studies have shown that, unlike other antidepressants, tianeptine stimulates the uptake of serotonin and increases 5-hydroxyindoleacetic acid levels in the brain [131]. It does not have anticholinergic effects and is also devoid of any effect on the cardiovascular and neuroendocrine systems [159]. Tianeptine shows no affinity for neurotransmitter receptors and its effects do not seem to depend upon blockade of the neuronal dopamine transporter [160]. Repeated administration increases the responsiveness of the $alpha_1$-adrenergic system [161]. Recent hypotheses on tianeptine's mode of action however, have involved the modulation of excitatory amino acid transmission and new evidence indicates that this antidepressant seems to specifically target the phosphorylation-state of glutamate receptors at the CA3 synapse [151]. Remarkably, tianeptine abolished stress-induced reduction of hypothalamic CRF concentration and markedly reduced stress-related increase of plasma ACTH and corticosterone concentrations[147]. These results suggest that the hypothalamus represents a primary target for antidepressants, a view also supported by the ability of tianeptine to attenuate, in stressed animals, the activation of the HPA axis [147,162]. Tianeptine-induced reduction of hypothalamic-pituitary-adrenal response to stress may constitute one of the mechanisms by which this drug antagonizes stress-induced behavioral deficits as well as prevents atrophy of neuronal dendrites [54,145,163].

Dosage. Tianeptine, kindly provided by the Institut de Recherches Internationales Servier (Paris, France), was dissolved in saline (0.9% NaCl) at a concentration of $10mg*ml^{-1}$, and injected intraperitoneously (i.p.) at the dosage of $10mg*kg^{-1}*day^{-1}$, for a 21 day-period, 30-45 minutes before exposure to the footshock procedure. The daily dose of $10mg*kg^{-1}*day^{-1}$ was chosen based on the indications provided by the drug manufacturer and literature review, as the effective dosage sufficient to prevent behavioral and neurochemical abnormalities in a chronic setting [54,147,151,164-166].

Reboxetine. Reboxetine is a potent and selective norepinephrine reuptake inhibitor without any affinity for neurotransmitter receptors that displays an antidepressant profile in both animal tests and in clinical trials. Unlike desipramine or imipramine, reboxetine has weak affinity for muscarinic, histaminergic H_1, adrenergic $alpha_1$, and dopaminergic D_2 receptors and low toxicity in animals. It is a mixture of (R,R) and (S,S) enantiomers, the latter being more potent although no qualitative difference in pharmacodynamic properties are observed between the two. Humans rapidly absorb reboxetine (t_{max} about 2 hours) with a terminal half-life of elimination ($t_{1/2}$) of 13 hours [167]. *In vivo* action of reboxetine is entirely consistent with the pharmacological action of an antidepressant with preferential action at the norepinephrine reuptake site [168]. Reboxetine has been shown to be an effective first-line

treatment for patients with all grades of depression, effective in preventing relapse and recurrence, and in offering significant benefits in terms of relieving the impaired social functioning associated with depressive disorders [169].

Dosage. Reboxetine, a racemic mixture of R,R- and S,S-([2-[alpha [2-ethoxyphenoxy] benzyl]-morpholine sulfate]) and (+)-(S,S)-reboxetine methanesulfon, was kindly provided by Pharmacia B.V. (The Netherlands). The noradrenaline reuptake inhibitor was dissolved in saline (0.9% NaCl) at a concentration of 20mg*ml^{-1}, and injected intraperitoneously (i.p.) at the dosage of 20mg*kg^{-1}*day^{-1}, for a 21 day-period, 30-45 minutes before exposure to the footshock procedure. The daily doses of 20mg*kg^{-1}*day^{-1} was chosen due to its reported low half-life and on the basis of the review of recent preclinical studies investigating the behavioral and neurochemical changes induced by prolonged administration of this antidepressant using similar dosages in rats [170-173].

Route of administration

In contrast to other studies in which osmotic pumps were used to deliver the drugs, here intraperitoneous injections were chosen as the route of administration. Although osmotic pumps allow a more constant administration, the concentration of antidepressant loaded into each pump must be estimated in advance on the basis of each individual animal's weight. The experimental design used here however presents some problems in establishing body weight since use was made of cyclic female rats and antidepressant treatment has been shown to modify female body weight growth by reducing food intake and, consequently, body weight gain [174,175]. Furthermore, we used a chronic aversive paradigm, which has been reported to strongly affect normal weight gain of animals [176]. As mentioned before, most preclinical studies, including pharmacological testing, have been performed in males, leaving females' response to stress and long-term antidepressant administration poorly explored. The use of osmotic pumps provides a reliable way of administration in males since their responses (in particular body weight changes during a long-term experiment) to different experimental conditions have been well defined. However, as we were unable to reliably predict body weight changes in cyclic female rats during chronic stress and long-term antidepressant administration, we decided to deliver the drugs by i.p. injections to guarantee a fixed and constant dosage throughout the entire duration of the experiment, independent of the effects of experimental conditions on the body weight of the individual animal.

Physiological and neuroendocrine correlates of the chronic stress response

To define the dynamic of the response to prolonged footshock stress and antidepressants, various physiological and neuroendocrine parameters were measured. Weight gain was monitored on a daily basis throughout the experiment, and upon termination, adrenal glands and thymus were removed and weighed. Graphs were constructed to serve as a reference to verify the severity of stress perceived by the animals. In addition, blood samples

were drawn by transcardial puncture immediately upon termination and stored at -80°C. These samples were then used to determine plasma corticosterone concentrations with HPLC.

Corticosterone: extraction and chromatography. For the assay, dexamethason was used as internal standard. After addition of the internal standard, plasma was extracted with 3 ml of diethylether, vortexed for 5 min and then centrifuged for 5 min at 3000 x *g*. The extraction procedure was repeated twice. The organic phase was evaporated to dryness in a 50°C waterbath. The residue was reconstituted with 200µL of mobile phase and 50µL was injected into the HPLC system. The mobile phase (flow rate 1.0mL/min) for the determination consisted of acetonitrile in ultrapure water (27:73 v/v). The concentration of both corticosterone and the internal standard was determined with UV detection at a wavelength of 254nm. The detection limit of the method was 10nM.

Histological procedure

Two hours after the beginning of the final session, the rats were terminated with an overdose of halothane which preceded a transcardial perfusion with 4% paraformaldehyde solution in 0.1M sodium phosphate buffer (pH 7.4). The brains were carefully removed and post-fixed in the same solution overnight at 4°C, before being transferred to a potassium phosphate buffer (KPBS 0.02 M, pH 7.4) and stored at 4°C. Following cryoprotection of the brains by overnight immersion in a 30% glucose solution, coronal serial sections of 40 µm were prepared on a cryostat microtome. Sections were collected in KPBS with sodiumazide and stored at 4°C.

Immunohistochemistry

The staining was performed on free-floating sections under continuous agitation. The sections were preincubated in 0.3% H_2O_2 for 15 min to reduce endogenous peroxidase activity, before being incubated in primary polyclonal rabbit anti-FOS (Oncogene Research Products, brands of CN Biosciences, Inc, an affiliate of Merck KGaA, Darmstadt, Germany; 1:10000 dilution in KPBS 0.02 M, pH 7.4) or anti-phospho-CREB antibodies (Upstate Biotechnology, Charlottesville, VA, USA: www.upstatebiotech.com; 1:1000 dilution in KPBS 0.02 M, pH 7.4) for 60-72 hr at 4°C. Subsequently, sections were washed with KPBS and incubated at room temperature with biotinylated goat anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA, USA; 1:1000 dilution) followed by ABC complex (Vector ABC kit, Vector Laboratories, Burlingame, CA, USA). After another wash, the reaction product was visualized by adding diaminobenzidine as chromogen and 1% H_2O_2 for 15 min. Then, the sections were washed, mounted on slides, dehydrated and coverslipped with DePex.

Quantification and data analysis

c-fos and phospho-CREB-labeled nuclei were quantified using a computerized imaging analysis system by an observer who was blind to group assignment. The quantification of the immunoreactive cell nuclei was performed using at least 4-5 sections per each brain area examined. The selected area from regions of interest (ROI) were digitized by using a Sony (SONY Corporation, Tokyo, Japan) charge-coupled device digital camera mounted on a

LEICA Leitz DMRB microscope (LEICA, Wetzlar, Germany) at x100 magnification. ROIs included the medial orbitofrontal cortex (mORB: Bregma +4.85 to +3.60), the medial prefrontal cortex (prelimbic (PrL) and the infralimbic area (InfraL); mPFC: Bregma +3.60 to +1.70), the anterior (AC: Bregma +3.20 to +0.95), and the posterior cingulate cortex (postCING: Bregma +1.70 to -1.08); the hippocampal dentate gyrus (DG: Bregma -2.00 to –3.90) and the CA3 area (CA3: Bregma –2.45 to –4.60); the central (CeA: Bregma –1.53 to –2.85), the lateral (LaA: Bregma –2.00 to -3.70), and the basolateral nucleus of the amygdala (BslA: Bregma –1.78 to –3.25); the paraventricular thalamic nucleus (PVT: Bregma –1.33 to –3.90); the paraventricular hypothalamic nucleus (PVN: Bregma –1.08 to –2.00) [177]. ROIs were outlined with a digital pen. Each digitized image was individually set at a threshold to subtract the background optical density, and the numbers of cell nuclei above the background were counted by using the computer-based image analysis system LEICA (LEICA Imaging System Ltd., Cambridge, England). Only cell nuclei that exceeded a defined threshold were detected by the image analysis system and subsequent counts were reported as number of positive cells/0.1mm^2. Phospho-CREB and FOS positive cells with gray levels below the defined thresholds were thus classified as “negative”. This is important for the understanding of our results, since this method does not allow us to discriminate between negative nuclei with no immunoreactivity and nuclei with (too) low immunoreactivity. In other words this method is not suitable for determining absolute protein levels. All areas were measured bilaterally (no left-right asymmetry of FOS or phospho-CREB immunoreactivity was found). *F* tests of variance were run on numbers of immunoreactive cell nuclei from individual brain regions from experimental and control conditions. That value determined whether *t* tests for equal or unequal variance were performed to compare the cell counts from individual brain regions of control and experimental conditions. $P < 0.05$ was defined as the level of significance between groups.

Results

Physiological and neuroendocrine correlates of the chronic stress response

Physiological parameters, including body weight gain as well as adrenal and thymus size, were measured throughout the experiment or upon termination.

Body weight gain

Vehicle. Body weights were measured daily during the chronic stress procedure. No differences were detected between CTR-vehicle and STR-vehicle females (fig. 1a). This finding is in accordance with previous preclinical data reporting that stress exposure does not affect weight gain in female rats as much as it does in males [178]. Although the lack of difference between CTR-vehicle and STR-vehicle groups, we decided to include this information anyway since the disruption of body weight regulation represents one of the most common side-effects of pharmacotherapy [174,175].

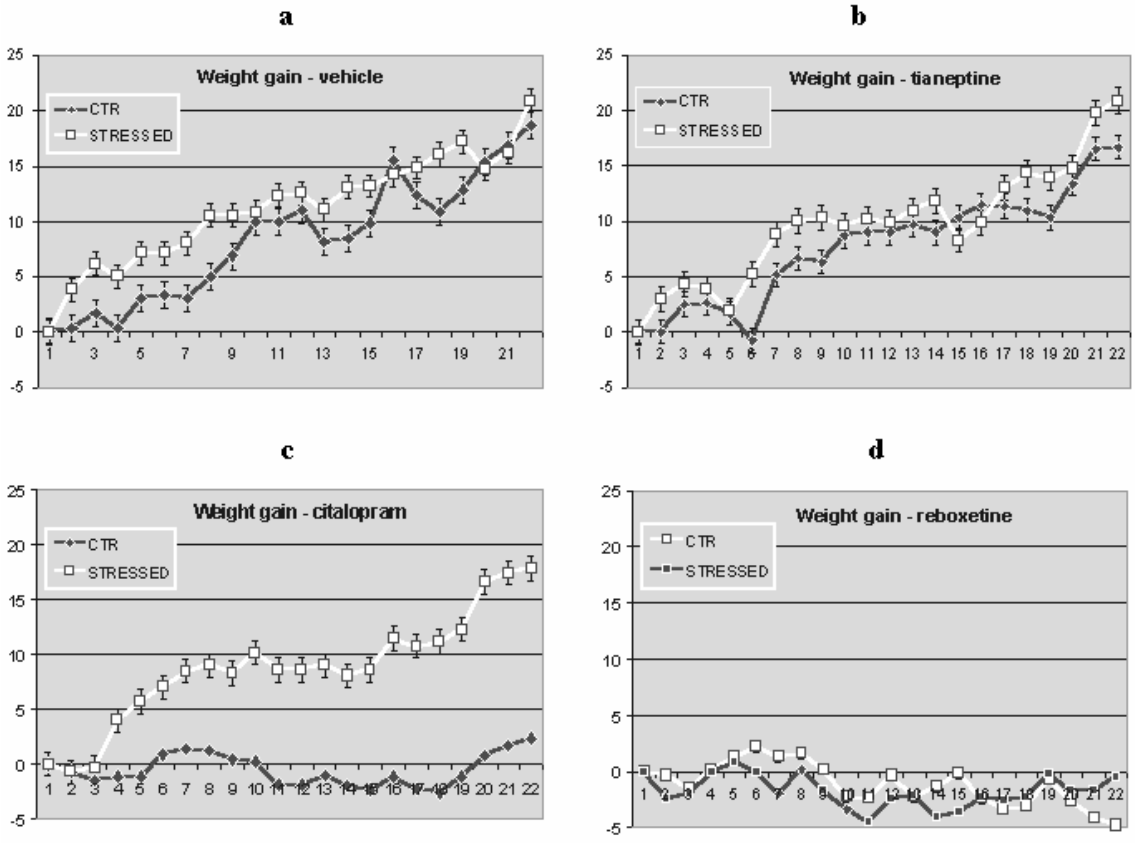


Figure 1. Body weight changes following prolonged stress and/or antidepressant administration.

Tianeptine. No changes in body weight gain were found between CTR-tianeptine and STR-tianeptine animals as well as between tianeptine- and vehicle-treated rats. Tianeptine thus

appears to carry out its action without influencing the normal curve of body weight growth (fig. 1b).

Citalopram. SSRI treatment resulted, in non-stressed females, in a significantly decreased body weight gain ($F=26.8$, $p<<0.001$; CTR-vehicle vs. CTR-SSRI). Serotonin plays a central role in food intake and body weight regulation, especially in the hypothalamus. Long-term SSRI administration has been shown to gradually desensitize the hypothalamic post-synaptic 5-HT_{1A} receptors [179] and this desensitization may affect food intake and weight gain regulation, leading to reduced body weight growth [180]. Surprisingly, chronic stress counteracted this effect ($F=11.06$, $p<0.007$; CTR-SSRI vs. stress-SSRI) restoring a normal weight gain curve (fig. 1c).

Reboxetine. A consistent reduction in body weight gain was observed immediately following the initiation of reboxetine treatment, both in stressed and non-stressed females, compared to vehicle-treated animals. These differences increased gradually throughout the experiment reaching highest significance at the end ($F=45.75$, $p<<0.001$, CTR-vehicle vs. CTR-reboxetine; $F=24.47$, $p<<0.001$, STR-vehicle vs. STR-reboxetine) (fig. 1d).

Plasma corticosterone concentration

Vehicle. No significant differences in corticosterone concentrations were found between any experimental groups with the only exception of STR-reboxetine animals (fig. 2a). Plasma samples were collected only at the time of death, occurring approximately 120 minutes after the final exposure to CSs. Plasma corticosterone levels, in response to stressful conditions, have been reported to reach a peak about 15-30 minutes following the threat and return to basal level in 60-90 minutes [181,182]. Chronically stressed rats however, showed an increased baseline plasma corticosterone concentration compared to control females (+22%).

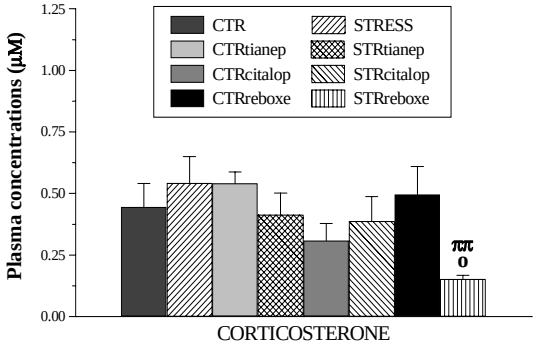


Figure 2a. Plasma corticosterone concentrations

Tianeptine. Although a slight increase of basal corticosterone concentration was observed following tianeptine treatment in non-stressed females (+22%), long-term antidepressant administration prevented the increased glucocorticoid levels detected following chronic footshock exposure (-24%, STR-vehicle vs. STR-tianeptine) (fig. 2a).

Citalopram. Both groups receiving long-term citalopram treatment showed lower basal corticosterone concentrations compared to CTR-vehicle (-69%) and STR-vehicle rats (-40%), respectively (fig. 2a).

Reboxetine. While CTR-reboxetine rats illustrated slightly increased basal serum corticosterone concentrations compared to CTR-vehicle animals (+11%), a significant reduction of plasma glucocorticoid levels was detected in STR-reboxetine females compared

to both STR-vehicle (-72%; F=12.74, p<0.012) and CTR-reboxetine group (-69%; F=21.51, p<0.01) (fig. 2a).

Adrenal and thymus weights

Vehicle. Chronic footshock stress caused marked adrenal hypertrophy (F=6.23, p<0.032) and a slight reduction of thymus weight (F=2.56, p<0.017) (fig. 2b,c).

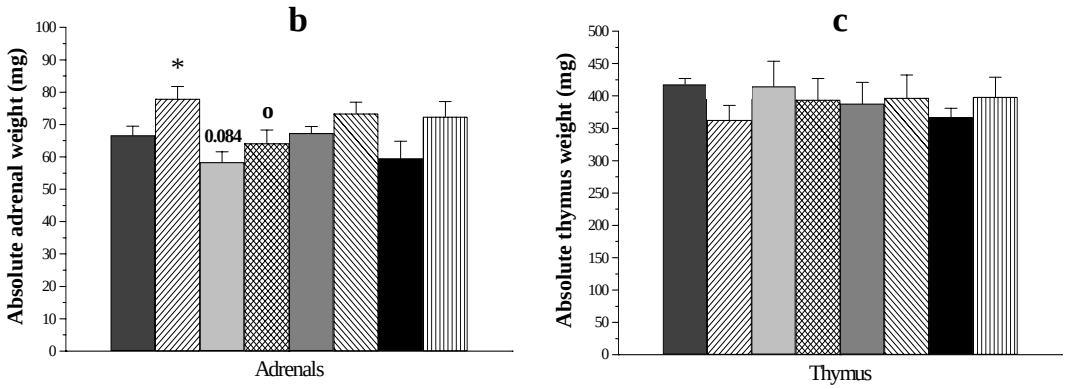


Figure 2b,c. Adrenal and thymus weight following chronic stress and antidepressant treatments

Tianeptine. Preclinical evidence suggests the HPA axis as one of tianeptine targets [147]. In support of this assumption, a marked reduction of adrenal gland weight was observed in non-stressed females following long-term antidepressant treatment (F=3.69, p<0.084, CTR-vehicle vs. CTR-tianeptine). More importantly, tianeptine administration significantly prevented the increased adrenal size seen in response to chronic footshock exposure (F=6.20, p<0.034, STR-vehicle vs. STR-tianeptine). No changes were observed after antidepressant treatment in thymus weight, both in non-stressed and stressed animals (fig. 2b,c).

Citalopram. Long-term SSRI treatment attenuated chronic footshock-induced adrenal hypertrophy. Chronically stressed rats treated with citalopram, in fact, reported a marked but not-significant enlargement of adrenal glands compared to non-stressed animals (F=2.03, p<0.18, CTR-citalopram vs. STR-citalopram; F=1.70, p<0.22, CTR-vehicle vs. STR-citalopram). Citalopram administration did not significantly affected thymus weight (fig. 2b,c).

Reboxetine. Although reboxetine treatment seems able to reduce adrenal weight in non-stressed females compared to vehicle-treated rats (F=1.26, p<0.29), this antidepressant attenuated stress-induced adrenal hypertrophy (F=3.37, p<0.10, CTR-reboxetine vs. STR-reboxetine). Interestingly, reboxetine alone without concurrent exposure to stressful conditions led to a marked reduction of thymus weight (F=2.89, p<0.12, CTR-vehicle vs. CTR-reboxetine). Remarkably, cyclic females, exposed simultaneously to stress and antidepressant treatment, showed only a slight and non-significant thymus weight reduction (F=0.77, p<0.40, CTR-vehicle vs. STR-reboxetine) (fig. 2b,c).

Immunohistochemistry

In the present study, changes of FOS and phospho-CREB immunoreactivity were examined in several cortical and subcortical regions, including the frontal cortex, the hippocampus, the amygdala, the thalamus, and the hypothalamus (fig. 3).

Chronic stress-induced neurochemical changes

Vehicle

FOS-ir. Chronically stressed females showed marked immunohistochemical changes in both cortical and limbic structures, including a significant increase of FOS-ir in the medial orbitofrontal cortex ($F=5.08$, $p<0.048$) (fig. 4a), the prelimbic cortex ($F=5.37$, $p<0.043$) (fig. 4b), the central nucleus of the amygdala ($F=33.09$, $p<<0.001$) (fig. 4h), and the paraventricular nucleus of the hypothalamus ($F=29.83$, $p<<0.001$) (fig. 4l). In contrast, an opposite and significant reduction of FOS-ir was observed in the hippocampal dentate gyrus ($F=5.02$, $p<0.05$) (fig. 4f).

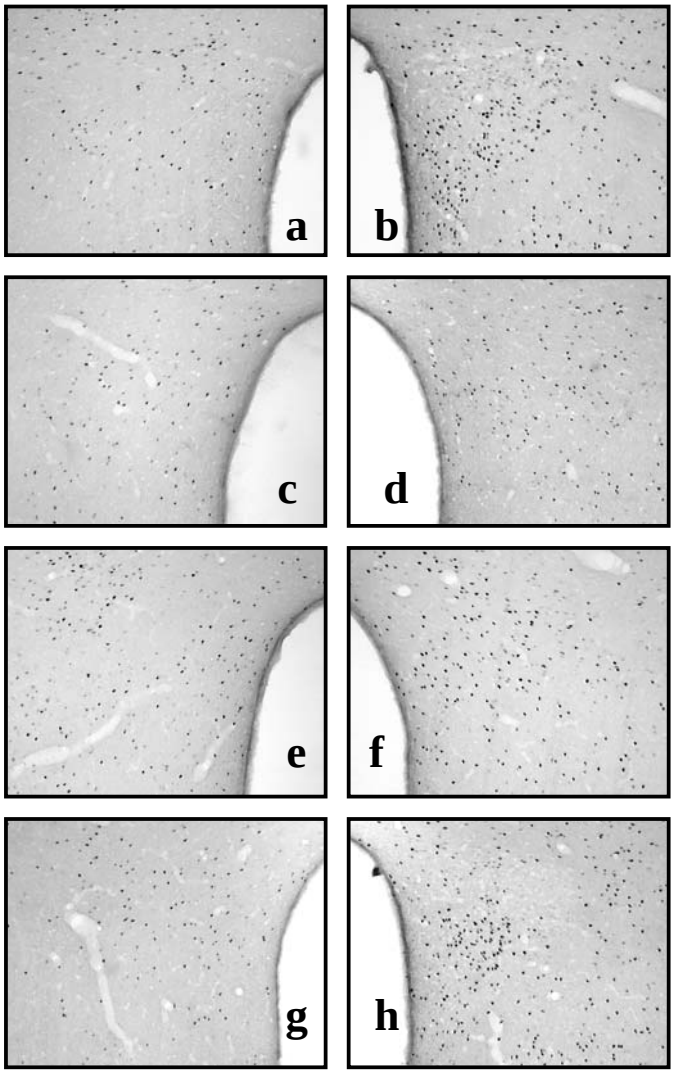


Figure 3. FOS-ir in the PVN following stress and antidepressant administration

Phospho-CREB immunoreactivity. Phospho-CREB expression was examined in cortical and subcortical areas including the medial orbitofrontal, the prelimbic, the infralimbic, the anterior cingulate, the posterior cingulate cortex, the lateral and the basolateral amygdala, the hippocampal dentate gyrus (fig. 5). A marked decreased CREB phosphorylation was observed, following prolonged footshock exposure, in the medial orbitofrontal cortex ($F=4.15$, $p<0.072$) (fig. 6a), the prelimbic cortex ($F=15.01$, $p<0.004$) (fig. 6b), the anterior cingulate cortex ($F=8.93$, $p<0.015$) (fig. 6d), and the DG ($F=10.76$, $p<0.01$) of cyclic female rats (fig. 6f).

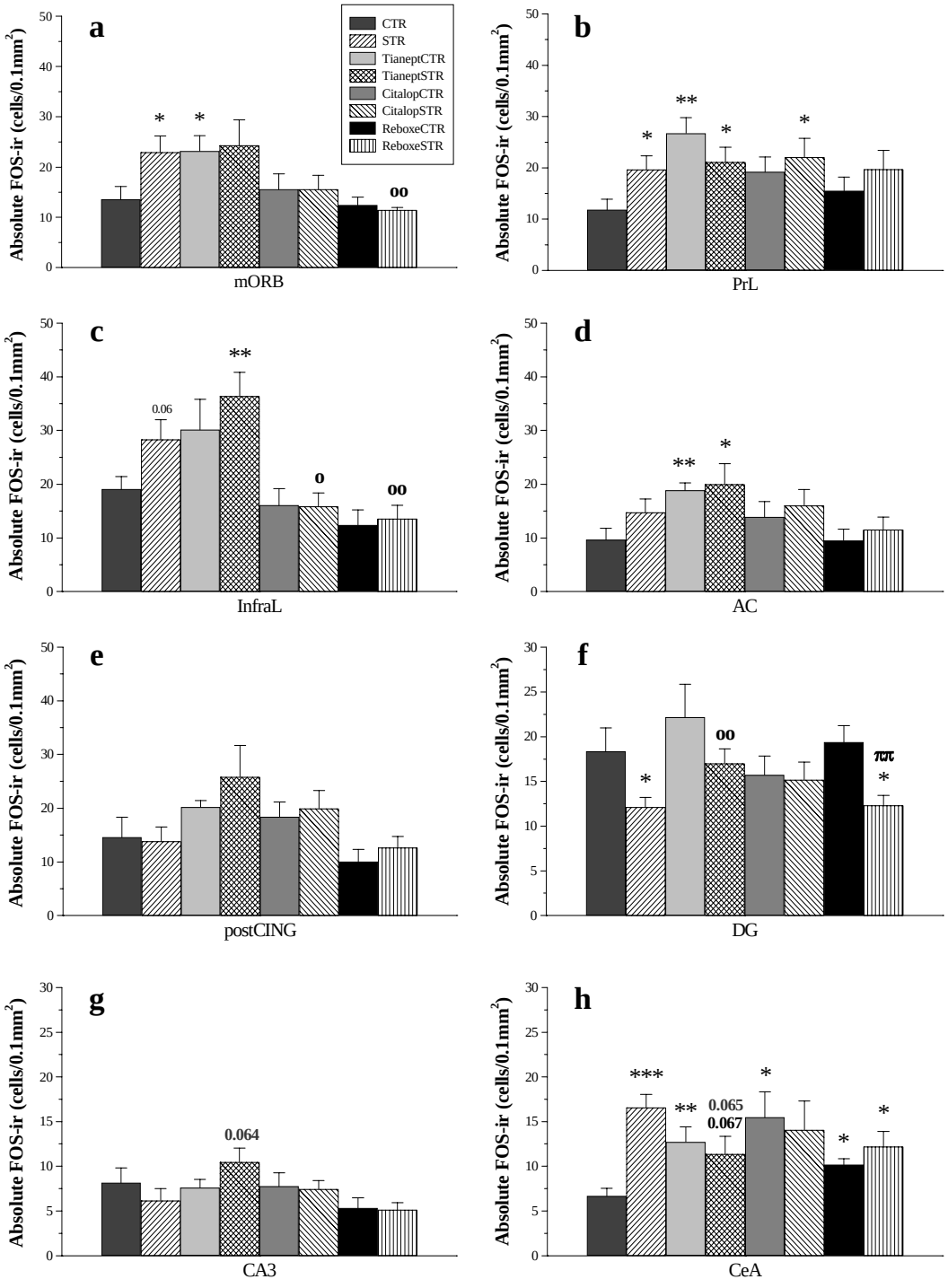
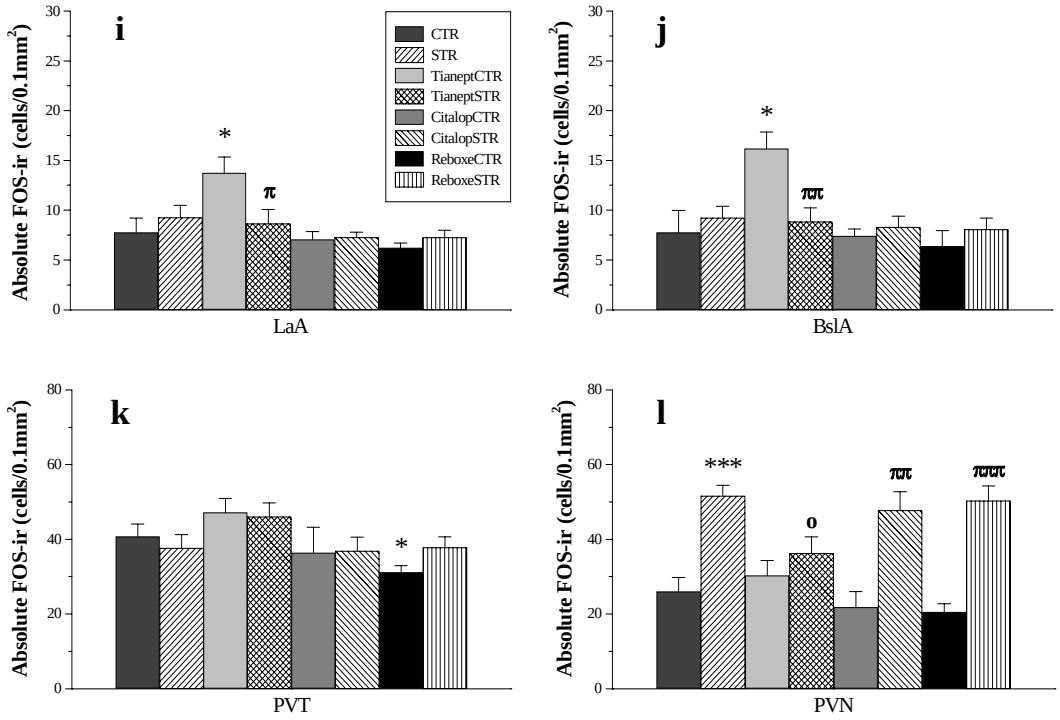


Figure 4. Effect of stress and/or concurrent antidepressant administration on absolute FOS-ir in: a-e) medial prefrontal cortex; f-g) hippocampus; h-j) amygdala; k) thalamus; l) hypothalamus. The symbol * expresses the comparison with CTR-vehicle (*=p<0.05; **=p<0.01; ***=p<0.001). The symbol ^{o} expresses the comparison between STR-vehicle and STR-antidepressant (^{o}=p<0.05; ^{oo}=p<0.01; ^{ooo}=p<0.001). The symbol $^{\pi}$ expresses the comparison between CTR-antidepressant and STR-antidepressant ($^{\pi}$=p<0.05; $^{\pi\pi}$=p<0.01; $^{\pi\pi\pi}$=p<0.001).



Long-term antidepressant administration on basal FOS-ir and CREB phosphorylation (CTR-vehicle vs. CTR-antidepressant)

Tianeptine

FOS-ir. Long-term tianeptine administration caused, in non-stressed animals, a significant increase of basal FOS-ir in the medial orbitofrontal cortex ($F=5.79$, $p<0.037$) (fig. 4a), the prelimbic cortex ($F=16.44$, $p<0.0023$) (fig. 4b), the anterior cingulate ($F=12.96$, $p<0.0049$) (fig. 4d), the central ($F=10.07$, $p<0.01$) (fig. 4h), the lateral ($F=7.79$, $p<0.019$) (fig. 4i), and the basolateral amygdala ($F=9.22$, $p<0.013$) (fig. 4j) compared to vehicle-treated females.

Phospho-CREB immunoreactivity. No significant regional changes in the level of basal cortical-limbic CREB phosphorylation were reported, in non-stressed animals, in response to long-term tianeptine treatment.

Citalopram

FOS-ir. Long-term citalopram administration did not significantly change the level of basal FOS-ir. The only exception was represented by the central nucleus of the amygdala where a marked increase of FOS-ir was observed after prolonged SSRI administration compared to vehicle-treated females ($F=8.70$, $p<0.015$) (fig. 4h).

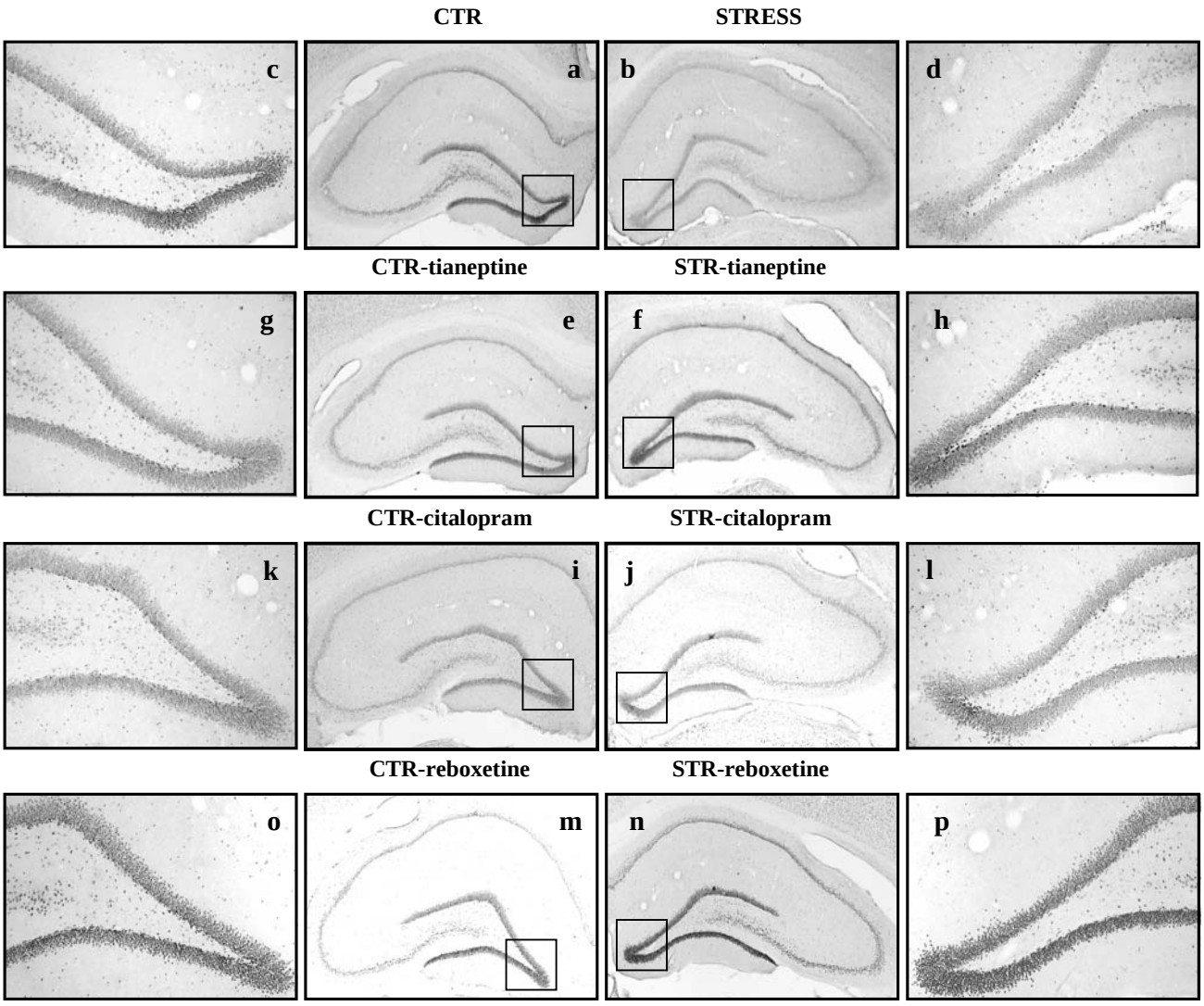


Figure 5. Hippocampal phospho-CREB immunoreactivity following prolonged stress and long-term antidepressant treatments.

Phospho-CREB immunoreactivity. Long-term citalopram treatment resulted in a marked reduction in the level of basal phospho-CREB immunoreactivity (fig. 6). Citalopram-treated females showed a significant reduction of CREB phosphorylation in the posterior cingulate cortex ($F=7.22$, $p<0.023$) (fig. 6e), the DG ($F=17.58$, $p<0.0019$) (fig. 6f), and the BslA ($F=7.53$, $p<0.021$) compared to vehicle-treated rats (fig. 6i).

Reboxetine

FOS-ir. Long-term reboxetine administration caused a significant increase of basal FOS-ir in the CeA ($F=9.74$, $p<0.011$) (fig. 4h), while a reduction was observed in the PVT ($F=6.35$, $p<0.03$) compared to vehicle-treated rats (fig. 4k).

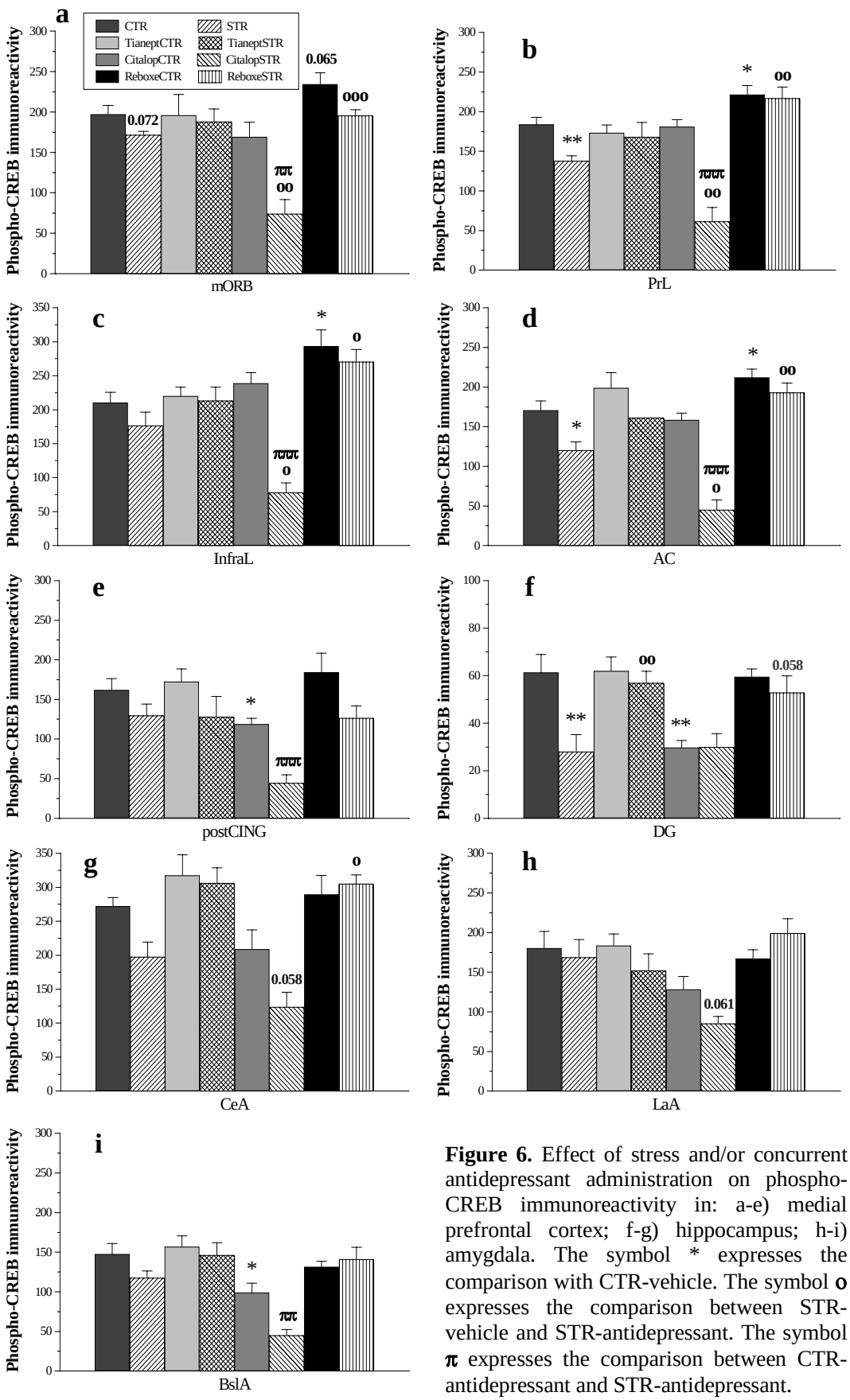


Figure 6. Effect of stress and/or concurrent antidepressant administration on phospho-CREB immunoreactivity in: a-e) medial prefrontal cortex; f-g) hippocampus; h-i) amygdala. The symbol * expresses the comparison with CTR-vehicle. The symbol **o** expresses the comparison between STR-vehicle and STR-antidepressant. The symbol **π** expresses the comparison between CTR-antidepressant and STR-antidepressant.

Phospho-CREB immunoreactivity. Long-term reboxetine treatment markedly enhanced basal cortical-limbic phospho-CREB immunoreactivity. This effect was particularly evident in the medial orbitofrontal cortex ($F=4.30$, $p<0.065$) (fig. 6a), the prelimbic cortex ($F=6.68$, $p<0.027$) (fig. 6b), the infralimbic cortex ($F=8.65$, $p<0.015$) (fig. 6c), and the anterior cingulate cortex ($F=6.88$, $p<0.025$) (fig. 6d).

Immunohistochemical changes induced by chronic stress and concurrent long-term antidepressant administration (STR-vehicle vs. STR-antidepressant)

Tianeptine

FOS-ir. Although long-term tianeptine administration did not normalize chronic stress-induced increased FOS-ir in prefrontocortical regions (fig. 4a-e), this antidepressant was effective in reversing stress-induced immunohistochemical changes in the hippocampal dentate gyrus ($F=5.90$, $p<0.033$) (fig. 4f) and CA3 area ($F=4.25$, $p<0.064$) (fig. 4g).

Phospho-CREB immunoreactivity. Long-term tianeptine administration effectively attenuated footshock-mediated reduction of phospho-CREB immunoreactivity in the DG ($F=13.32$, $p<0.0045$) (fig. 6f) and partially in prefrontocortical areas (fig. 6a-e). No significant immunohistochemical changes were observed in the amygdala (fig. 6g-i).

Citalopram

FOS-ir. Long-term citalopram administration was able to alleviate FOS-ir changes observed in response to chronic footshock exposure. This beneficial effect was particularly evident in the medial orbitofrontal cortex (fig. 4a), the infralimbic cortex ($F=8.22$, $p<0.015$) (fig. 4c), the dentate gyrus (fig. 4f) and the central amygdala (fig. 4h). The only exception was represented by the prelimbic cortex where a significantly increased FOS expression was observed in STR-citalopram rats compared to CTR-vehicle females ($F=5.22$, $p<0.043$) (fig. 4b). However, markedly increased activity was also found in CTR-SSRI animals suggesting that stimulation of serotonergic neurotransmission might have been the main factor responsible for this higher activation. Interestingly, STR-SSRI females illustrated significantly increased FOS-ir in the paraventricular hypothalamic nucleus compared to both CTR-vehicle ($F=11.58$, $p<0.006$) and CTR-SSRI rats ($F=15.36$, $p<0.0024$) (fig. 4l).

Phospho-CREB immunoreactivity. Long-term citalopram administration was not able to attenuate CREB phosphorylation changes associated with chronic footshock exposure. Remarkably, STR-SSRI females reported a marked reduction of phospho-CREB immunoreactivity that was, in several regions, even larger than that detected in both STR-vehicle and CTR-SSRI animals (fig. 6), including the mORB ($F=21.52$, $p<0.0017$, STR-vehicle vs. STR-SSRI; $F=12.18$, $p<0.007$, CTR-SSRI vs. STR-SSRI) (fig. 6a), the PrL ($F=14.2$, $p<0.0044$; $F=32.25$, $p<<0.001$) (fig. 6b), the InfraL ($F=5.7$, $p<0.041$; $F=49.18$, $p<<0.001$) (fig. 6c), the AC ($F=7.43$, $p<0.023$; $F=48.11$, $p<<0.001$) (fig. 6d), the posterior cingulate ($F=29.10$, $p<<0.001$, CTR-SSRI vs. STR-SSRI) (fig. 6e), the CeA ($F=4.69$, $p<0.058$, CTR-SSRI vs. STR-SSRI) (fig. 6g), the LaA ($F=4.59$, $p<0.061$, CTR-SSRI vs. STR-SSRI) (fig. 6h), and the BslA ($F=12.20$, $p<0.0068$, CTR-SSRI vs. STR-SSRI) (fig. 6i).

Reboxetine

FOS-ir. Long-term reboxetine administration attenuated footshock-induced changes, significantly reducing stress-induced FOS upregulation in the mORB (STR-vehicle vs. STR-reboxetine, $F=14.21$, $p<0.0031$) (fig. 4a) and InfraL ($F=11.72$, $p<0.0057$) (fig. 4c) but only partially in the CeA ($F=3.59$, $p<0.085$) (fig. 4h). Antidepressant treatment did not prevent the significant chronic footshock-induced reduction of FOS-ir in the DG ($F=5.17$, $p<0.044$, CTR-vehicle vs. STR-reboxetine; $F=11.51$, $p<0.006$, CTR-reboxetine vs. STR-reboxetine) (fig. 4f) or the increased neuronal activation detected in the PVN ($F=19.35$, $p<0.0011$, CTR-vehicle vs. STR-reboxetine; $F=39.48$, $p<<0.001$ CTR-reboxetine vs. STR-reboxetine) (fig. 4l).

Phospho-CREB immunoreactivity. Interestingly, prolonged reboxetine treatment normalized chronic footshock-mediated reduction of CREB phosphorylation in the medial orbitofrontal cortex (STR-vehicle vs. STR-reboxetine, $F=35.86$, $p<<0.001$) (fig. 6a), the prelimbic cortex ($F=17.87$, $p<0.0024$) (fig. 6b), the infralimbic cortex ($F=9.41$, $p<0.013$) (fig. 6c), the anterior cingulate cortex ($F=15.11$, $p<0.0037$) (fig. 6d), the dentate gyrus ($F=4.59$, $p<0.058$) (fig. 6f), and the central nucleus of the amygdala ($F=15.34$, $p<0.0035$) (fig. 6g).

*Chronic stress-induced neuroendocrine and immunohisto-chemical changes in the female brain: role of long-term citalopram treatment**

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***Adapted from the manuscript submitted to "Neuropsychopharmacology"**

In the present study, an attempt was made to define the neuroendocrine and immunohistochemical changes induced by prolonged footshock exposure and/or concomitant long-term antidepressant treatment in cyclic female rats. FOS-ir has been widely used as a molecular marker of neuronal activity [181,183,184] and changes in expression of this immediate early gene have been used to explore the neurocircuits underlying various higher-order processes including learning, memory, affective style, emotion and stress response regulation [184-186]. Phospho-CREB immunoreactivity, instead, was used as molecular correlate of neuronal plasticity [105,187,188]. Increased CREB phosphorylation, especially in the amygdala and hippocampus, has been reported to be critical for cognitive processing [122,189-193]. By making use of the properties of these two cellular markers, we investigated the impact of sustained footshock stress on neuronal functioning as well as the ability of long-term citalopram administration to attenuate stress-induced alterations in the female rat brain.

Exposure to chronic and severe stress was confirmed by marked neuroendocrine changes, including increased basal corticosterone levels, adrenal hypertrophy (fig. 2b), reduced thymus weight (fig. 2c), and enhanced FOS expression in the PVN (fig. 3, 4l). This PVN plays a key role in the modulation of HPA axis [194,195] and the increased activation detected in this nucleus following chronic stress (fig. 3, 4l) may illustrate a condition of functional hyperactivity. Interestingly, sustained footshock stress has been shown to strongly activate the HPA axis leading to elevated plasma glucocorticoid levels[196]. The increased PVN activation observed here (fig. 4l) together with the significant adrenal hypertrophy (fig. 2b) seem to substantiate this finding and document the lack of habituation and, more importantly, the persistent activation of HPA axis in response to repeated footshock stress. In addition to the PVN however, a markedly increased FOS-ir was also found in several other cortical and subcortical structures, such as the medial orbitofrontal cortex (fig. 4a), the prelimbic cortex (fig. 4b), the infralimbic cortex (fig. 4c), and the central amygdala (fig. 4h). In contrast, a reduced neuronal activity was found in the hippocampus (in both the dentate gyrus and the CA3 area) (fig. 4f,g). Furthermore, a significantly stress-induced decreased CREB phosphorylation was

observed in the prelimbic cortex (fig. 6b), the anterior cingulate cortex (fig. 6d), and the dentate gyrus (fig. 6f). It is tempting to speculate that these immunohistochemical changes may illustrate the detrimental influence of sustained stress on brain neurochemistry.

Experimental evidence has pointed to the hippocampus as one of the main target of glucocorticoid-mediated actions [90,197,198]. In line with the latter, our results show a significant reduction of FOS-ir (fig. 4f,g) and CREB phosphorylation (fig. 5, 6f) in the hippocampal dentate gyrus of chronically stressed females. It is of interest to note that selective chronic stress-related effects has been reported in the hippocampus of different animal species, including a significant reduction of cell proliferation and dendritic atrophy [54,137,199]. There is some consensus concerning the view that glucocorticoids represent a key mediator in these processes [54] and, for this reason, it is intriguing to relate the reduction of hippocampal FOS-ir (hypoactivity) and phospho-CREB expression (decreased neuronal plasticity) to stress-induced functional and/or morphological impairments. The reduction of CREB phosphorylation however, was not limited to the hippocampus but involved also other forebrain structures (fig. 6). Phosphorylated CREB modulates the transcription of several genes involved in the regulation of neuronal plasticity [187,200,201]. Chronic stress and prolonged exposure to elevated glucocorticoid levels have been reported to reduce both BDNF and CREB expression in the brain [114,124,126,176]. The results presented here, in accordance with previous findings, seem to support an inhibitory action of repeated footshock stress on CREB phosphorylation (fig. 6), an effect possibly related to the persistent activation of the HPA axis.

One possible way to reverse stress-induced abnormalities is by long-term antidepressant treatment [54,202,203]. An important aspect of antidepressants' clinical effectiveness is represented by their ability to attenuate some of the "state-related functional abnormalities" often observed in depressed subjects, such as reduced anterior cingulate and prefrontocortical activity [97,204-207]. It has been proposed that antidepressants, by reversing these state-related abnormalities, may help to correct specific cortical-limbic deficits involved in the development and/or maintenance of affective disorders [85,208]. It is important to mention that the normalization of stress-related dysfunctions constitutes a fundamental step for successful clinical recovery [209,210]. A common abnormality observed following prolonged stress exposure and reported by approximately 50% of depressed subjects is the hyperactivity of the HPA axis [149]. Interestingly, several studies have illustrated a beneficial effect of antidepressants in correcting this disturbance in both humans and animals [54,211,212]. In line with the latter, long-term citalopram administration (20 days, i.p., 20mg*kg^{-1}*day^{-1}), although unable to prevent stress-induced increased FOS-ir in the PVN (fig 4l), effectively reduced basal (-69%) and stress-related corticosterone levels (-40%) (fig. 2a) and, more important,

attenuated footshock-induced adrenal hypertrophy in female rats (fig. 2b). In addition, SSRI treatment partially normalized chronic stress-induced FOS-ir changes in key cortical and subcortical regions primarily involved in the coordination of HPA axis activity, such as the medial orbitofrontal cortex (fig. 4a), the infralimbic cortex [213,214] (fig. 4c), the dentate gyrus [215,216] (fig. 4f), and the central amygdala [217-219] (fig. 4h). Cross-talks between prefrontocortical and limbic structures play a critical role in the modulation of HPA axis activity and the disruption of these coordinated cortical-limbic interactions has been suggested as a central mechanism involved in the development of abnormal stress response regulation [149]. Antidepressants, on the other hand, have been proposed as potential candidates to correct this dysfunction by desensitizing the HPA axis [152]. Since chronically stressed females concurrently treated with citalopram demonstrated an increased PVN FOS-ir (fig. 4l) but no adrenal hypertrophy (fig. 2b), it is intriguing to speculate that long-term antidepressant administration helped to reestablish a coordinated HPA axis regulation and, consequently, limit the adverse effects associated with the persistent stress-induced elevation of glucocorticoid levels. SSRIs have been reported to gradually desensitize the hypothalamic post-synaptic 5-HT_{1A} receptors [179], crucially involved in the regulation of CRF release [220]. One may contemplate whether this citalopram-induced desensitization and reduced CRF secretion might contribute to the decrease of basal and stress-related corticosterone levels, therefore reducing the adrenal hypertrophy otherwise observed following repeated footshock exposure (fig 2b). Although SSRI treatment seemed to attenuate the overall response of the HPA axis, citalopram did not inhibit the significant increase of PVN FOS-ir in chronically stressed females. This finding however, is consistent with previous reports documenting the participation of many different neurotransmitters in the modulation of various aspects of the stress response [221]. An intriguing possibility is that this antidepressant might prevent the development of HPA axis hyperactivity, not by desensitazing hypothalamic receptors but by attenuating the occurrence of selective abnormalities in cortical-limbic regions involved in the modulation of this stress response system. The latter may be illustrated by the ability of citalopram to prevent / reverse stress-induced FOS-ir changes in the prefrontal cortex, the amygdala, and the hippocampus. Prolonged citalopram administration may thus protect the brain against the deleterious effects of abnormal HPA axis modulation and persistently elevated glucocorticoid levels by limiting stress-mediated increase of stimulatory input from the amygdala to the hypothalamus and avoiding footshock-induced disruption of central feedback inhibition governed by the prefrontal cortex and hippocampus.

An additional mechanism underlying the therapeutic action of antidepressants involves the attenuation of structural alterations observed in depressed subjects, such as dendritic atrophy and neuronal pathology [95,98]. Remarkably, structural deficits similar to those observed in depression have also been reported in chronically stressed

animals[54,137]. As mentioned above, repeated stress was associated with a marked reduction of CREB phosphorylation, particularly in the medial orbitofrontal cortex (fig. 6a), the prelimbic cortex (fig. 6b), the anterior cingulate (fig. 6d), and the hippocampal dentate gyrus (fig. 6f). Sustained HPA axis hyperactivity as well as persistently high glucocorticoid levels have been reported to inhibit the phosphorylation of this transcription factor [176,222], suggesting an abnormal activity of this stress response axis as a key factor in the development of morphological abnormalities. It is interesting to note that while several studies have emphasized the ability of the atypical antidepressant tianeptine to correct stress-induced structural defects [54,199], preclinical investigations evaluating the effects of TCAs and SSRIs have failed to reveal any beneficial action of these latter antidepressants [145]. The results presented here seem in line with these findings, as long-term citalopram treatment failed to prevent stress-induced reduction of phospho-CREB expression in cyclic female rats (fig. 6). Notably, citalopram administration strengthened stress-induced inhibition of CREB phosphorylation. The biological importance of this effect remains obscure. A possibility however may lie in the synergistic action between serotonin reuptake inhibition and glucocorticoids. Under severe stressful conditions, the interactions between elevated serotonin and glucocorticoid levels may represent a critical event in the process that leads to the reduction of neuronal plasticity and the development of stress-induced structural impairments [54]. Citalopram, by inhibiting serotonin reuptake, prolongs the presence of this neurotransmitter in the synaptic cleft. During chronic stress however, the simultaneous exposure to elevated serotonin and glucocorticoid concentrations may result in the combination of their individual effects and have deleterious consequences for neuronal plasticity. This synergistic action may result in greater reduction of phospho-CREB expression in cyclic female rats concurrently exposed to chronic footshock and SSRI treatment, especially in frontocortical territories (fig. 6a-e), compared to chronically stressed vehicle treated animals.

*Normalization of chronic stress-induced hippocampal-HPA axis dysregulation by long-term tianeptine administration**

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***Adapted from the manuscript submitted to "Molecular Pharmacology"**

As previously stated, prolonged HPA axis activation may lead to the development of structural abnormalities in the hippocampus and prefrontal cortex [87,90,197,198]. Preclinical studies however, have also suggested a critical role of other key players, besides glucocorticoids, in the modulation of negative effects of chronic stress on neuronal plasticity. Excessive glutamate exposure, for instance, has also been associated with neurotoxicity and dendritic atrophy [73,92,198]. Accordingly, pharmacological treatments selectively targeting glutamatergic neurotransmission have been reported to be able to effectively prevent the occurrence of stress-induced structural defects [73,74,223].

Tianeptine is an effective antidepressant that, in contrast to SSRIs and tricyclic agents, does not act through the inhibition of serotonin reuptake. On the contrary, one mechanism by which this atypical antidepressant is believed to exert its beneficial effect is by stimulating the removal of serotonin from neuronal terminals [150]. Recently however, some contention has emerged regarding tianeptine's mode of action, raising some doubts concerning its selectivity for serotonergic transmission. New preclinical data, for instance, seem to implicate the excitatory amino acid glutamate as an additional target of this drug [54,151]. It is of interest to note that serotonergic and glutamatergic systems are closely interconnected and superimposed. Moreover, serotonin plays a central role in the modulation of glutamate release [54]. Despite the specific nature of the molecular substrates underlying tianeptine's therapeutic action, this relationship between serotonin and glutamate remains of particular interest. The latter holds particularly true with respect to stressful conditions as these are associated with massive release of serotonin, glutamate, and corticosteroids [54]. Under sustained stressful conditions, prolonged interactions between stress hormones and neurotransmitters may thus present a serious neuronal caveat. It has been suggested that the simultaneous and persistent elevation of serotonin, glutamate, and glucocorticoids levels may facilitate the development of neuronal defects [54,83,88,224,225]. Through the removal of serotonin from the synaptic cleft and/or targeting the glutamatergic system, tianeptine may thereby attenuate the deleterious interaction between neurotransmitters and stress hormones and

the occurrence of structural defects such as dendritic atrophy and reduced granule cell proliferation.

Long-term tianeptine treatment resulted, in cyclic female rats, in a significant increase of basal FOS-ir in the medial orbitofrontal cortex (fig. 4a), the prelimbic cortex (fig. 4b), the anterior cingulate cortex (fig. 4d), the central (fig. 4h), the lateral (fig. 4i), and the basolateral amygdala (fig. 4j). These effects might be related to the "serotonin reuptake-enhancing" nature of this antidepressant. Long-term tianeptine administration appears to prevent footshock-induced immunohistochemical changes in the hippocampus and hypothalamus, supporting previous reports concerning the beneficial effect of this antidepressant on stress-induced neuronal abnormalities in males [54,199]. More important, chronically stressed females concomitantly treated with this atypical antidepressant showed a limited increase of FOS-ir in the PVN compared to both vehicle treated non-stressed and stressed animals (fig. 4l). In further support of this "anti-stress" effect, tianeptine markedly decreased adrenal weight in non-stressed females and, more importantly, prevented footshock-induced adrenal hypertrophy in chronically stressed animals (fig. 2b).

Prevention of stress-induced HPA axis hyperactivity seems to represent a fundamental step in tianeptine's mechanism of action. Whereas both acute and prolonged footshock exposure have been related to increased PVN and HPA axis activation (see chapter 1 and 2), long-term tianeptine administration significantly lowered stress-induced FOS-ir in this hypothalamic nucleus (fig. 4l) and prevented footshock-induced adrenal hypertrophy (fig. 2b). The pharmacological profile of this antidepressant, together with analysis of its neurochemical effects, may provide valuable insights into the mechanisms through which tianeptine regulates HPA axis function. While serotonin is known to play a central role in the modulation of HPA axis activity in response to stress [202,226,227], tianeptine-induced enhancement of serotonin reuptake may directly limit stress-induced HPA axis activation. Alternatively, prolonged antidepressant treatment may also interfere with glutamate action in the synaptic cleft[151]. Like serotonin, this excitatory amino acid is involved in the regulation of stress response[194,195,228], yet it is also a major candidate in the modulation of stress-induced hippocampal neurotoxicity [54,83,88,224,225]. By targeting simultaneously serotonin and glutamate neurotransmission, tianeptine may attenuate the deleterious effects associated with massive neurotransmitter release following stressful conditions. Besides the role played by serotonin and glutamate however, sustained exposure to high glucocorticoid concentrations represents another key factor in the development of neuronal defects [54,87]. Chronically elevated glucocorticoid levels may result from an abnormal modulation of HPA axis response, possibly caused by impaired hippocampal feedback inhibition [229-231]. Stress and glucocorticoid have been reported to reduce BDNF expression [125,126,232], a fundamental element in the regulation of hippocampal neurogenesis and plasticity. This

action may ultimately lead to dendritic atrophy and reduced hippocampal metabolism, disrupting the ability of this limbic structure to efficiently regulate HPA axis function. A possible molecular mechanism underlying tianeptine's beneficial action in the hippocampus involves the attenuation of stress-induced reduction of CREB phosphorylation (fig. 6f). Our results confirm the inhibitory influence of repeated footshock stress on CREB phosphorylation in both male [176] and female rats (fig. 6). Given the critical role of CREB in the modulation of BDNF transcription [81], this finding may provide a possible molecular pathway underlying stress-induced hippocampal structural defects. Long-term tianeptine treatment, on the other hand, significantly prevented stress-induced reduction of CREB phosphorylation in the dentate gyrus (fig. 6f). It is intriguing to speculate that tianeptine, by attenuating stress-induced reduction of neuronal plasticity, may prevent the development of structural and functional abnormalities in the hippocampus, restoring its ability to efficiently regulate the activity of the HPA axis and thereby limiting footshock-induced hyperactivity of this critical stress response system.

*Prevention of chronic stress-induced reduction of neuronal plasticity by long-term reboxetine treatment**

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Although in the past decade attention has been largely focused on the involvement of serotonin in the pathophysiology and treatment of depression, the abnormal activity of other neurotransmitter systems might underlie some of the functional and structural deficits reported in depressed subjects. As growing clinical literature has proven, affective disorders are not merely serotonin-related illnesses but diverse and complex pathologies involving dysfunctions in multiple neurotransmitter systems. Abnormalities of noradrenergic function, for instance, have already been established in depression and this neurotransmitter system is becoming an interesting candidate in the development of new and, hopefully, more efficient pharmacological treatments [168,233]. Interest in these compounds is mostly attributable to the central role of norepinephrine in the modulation of stress response [234-236] and strong strong evidence supporting the relationship between alterations in the noradrenergic system and behaviors of fear and anxiety [237,238]. These findings have thus generated the hypothesis that some of the symptoms seen in subjects with affective disorders may be related to abnormalities of noradrenergic neurotransmission [239-243]. Since abnormal noradrenergic regulation may participate to the development of stress-related psychiatric disorders, we decided to explore the neurochemical changes induced by long-term reboxetine administration and examine the ability of this antidepressant to correct chronic footshock-induced cortical-limbic alterations.

Reboxetine is a highly selective noradrenaline reuptake inhibitor and represents the first of a new generation of antidepressant agents with specificity for the noradrenergic system [168,233]. More importantly, the selectivity of this compound allows the study of neurochemical alterations induced by long-term stimulation of the noradrenergic system. Due to the recent introduction of reboxetine in clinical practice however, only a few preclinical studies have investigated the neurochemical alterations associated with its prolonged administration [171,173,212]. This lack of data has limited our understanding of the intracellular cascades involved in the modulation of the therapeutic effects of this antidepressant. Surprisingly, a reduction of cortical phospho-CREB expression has previously been documented in response to long-term reboxetine administration in rats [244]. This finding however, seems in conflict with various studies reporting a general increased CREB transcription and phosphorylation in different

cortical and subcortical structures, including the frontal cortex and the hippocampus, following long-term antidepressant treatments [76,77,117,119]. Our results, in non-stressed cyclic females, seem in line with these latter findings, as illustrated by the selectively enhancement of CREB phosphorylation, following chronic reboxetine administration, in the medial orbitofrontal (fig. 6a), the prelimbic (fig. 6b), the infralimbic (fig. 6c) and the anterior cingulate cortex (fig. 6d). No changes were found in other cortical or subcortical structures (fig. 6). The discrepancy between our and Manier's results remains unknown. However, while Manier and colleagues performed their study in male rats [244], we decided to carry out our investigation in cyclic female animals. This important methodological difference may thus account for the discrepancy observed between the two studies. Ovarian hormones have been reported to affect the intracellular pathways modulating synaptic plasticity [245-247] and stimulate the transcription of several genes including BDNF and tyrosine hydroxylase [248-253], the limiting enzyme in the biochemical cascade regulating norepinephrine synthesis. It is plausible that the presence of estrogen and/or progesterone might affect the immunohistochemical changes induced by long-term reboxetine administration. Current studies are being performed to investigate the role of ovarian hormones in the transduction of stress-related signals and the modulation of reboxetine-mediated neurochemical adaptations.

Long-term reboxetine treatment attenuated chronic footshock-induced increased FOS-ir in cortical regions, including the medial orbitofrontal (fig. 4a) and infralimbic cortex (fig. 4c). However, it did not normalize stress-induced FOS-ir changes in subcortical regions, as documented by the significant increased neuronal activity detected in the central amygdala (fig. 4h) and paraventricular hypothalamic nucleus (fig. 4l). The amygdala and the PVN, together with the locus coeruleus, represent key components of the noradrenergic stress response system [234,237,254]. It is of interest to note that the increased neuronal activity in the central amygdala following reboxetine treatment seems to be related to the stimulation of noradrenergic function caused by the norepinephrine reuptake-enhancing nature of the antidepressant, as both control and stressed rats reported a similar induction FOS-ir (fig. 4h). In contrast, the enhanced FOS-ir in the PVN appears to depend on the activation of this nucleus by exposure to threatening conditions and not upon the pharmacological profile of the drug, as the increased neuronal activity was found only in chronically stressed rats (fig. 4l). Moreover, long-term reboxetine treatment was not able to correct the significant reduction of FOS-ir detected in the hippocampus following repeated footshock stress (fig. 4f,g).

In addition to altered patterns of FOS-ir, norepinephrine reuptake inhibition also induced marked changes in the level of CREB phosphorylation. Chronically stressed females treated with reboxetine showed a significant enhancement of phospho-CREB expression in the medial orbitofrontal cortex (fig. 6a), the prelimbic cortex (fig. 6b), the

infralimbic cortex (fig. 6c), the anterior cingulate cortex (fig. 6d), the hippocampal dentate gyrus (fig. 6f), and the central nucleus of the amygdala (fig. 6g) compared to stressed animals only treated with vehicle. Long-term antidepressant administration also enhanced phospho-CREB immunoreactivity above baseline in various prefrontocortical areas such as the medial orbitofrontal cortex (fig. 6a), the prelimbic cortex (fig. 6b), the infralimbic cortex (fig. 6c), and the anterior cingulate cortex (fig. 6d). Remarkably, reboxetine administration reversed footshock-induced reduction of CREB phosphorylation in both cortical and subcortical structures (fig. 6). These findings seem thus to substantiate a positive action of reboxetine on neuronal plasticity, counteracting the detrimental influences of persistent footshock stress. Prolonged stress as well as sustained elevation of glucocorticoid levels have been reported to down-regulate BDNF expression [125,126,232]. Reboxetine, by reversing footshock-induced reduction of CREB phosphorylation, might attenuate stress-induced inhibition of BDNF expression. This may in turn provide selective cortical and subcortical structures, involved in the modulation of stress response, with the necessary plasticity needed to cope with persistent adverse conditions and avoid the development of abnormal HPA axis activity. It is also important to note that prevention of HPA axis hyperactivity is not obtained by desensitizing the PVN, as shown by the significant induction of FOS-ir in this hypothalamic nucleus in females simultaneously subjected to footshock and reboxetine treatment.

Conclusions and limitations

Limitations

Before drawing any final conclusions, one must first always consider the limitations of the experimental design. A few points of interest are discussed below.

A major point of consideration lies in the fact that, in this study, use was made of cyclic female rats. A confounding element in the interpretation of the results was represented by the hormonal state of the animals. Intracellular transduction cascades as well as the neurocircuits regulating stress response are prime targets for ovarian hormone action [255-257]. In turn, release of sex steroids is also influenced by adverse experiences [258]. Estrogen and progesterone may thus play a critical role in determining the immunohistochemical adaptations observed here following long-term footshock exposure and / or antidepressant treatment.

Although not a drawback of the experimental design, we have chosen not to address several issues. For instance, immunohistochemical changes induced by antidepressant treatments in midbrain regions such as the raphe nuclei and the locus coeruleus were not discussed here. As these regions contain the majority of serotonergic and noradrenergic cell bodies, these could provide important insights into the acute actions of stress and antidepressant treatments. Nevertheless, we preferred to focus on those alterations occurring in cortical and subcortical regions instead, since our goal was to investigate the neurobiological mechanisms underlying long-term stress exposure and / or antidepressant administration. Cortical-limbic regions, besides receiving strong serotonergic and noradrenergic projections, are also fundamentally involved in the regulation of stress, cognitive and emotional responses. They therefore most likely represent more suitable targets in the understanding of the molecular events involved in the development of neuronal impairments and the neurobiological mechanisms mediating antidepressant response.

An additional point of consideration regards the extent of this data analysis. In this paper we do not provide inter-drug comparisons (as stated earlier we used a 2x2 analysis) at the molecular level since our main objective was limited to exploring the ability of specific antidepressants to attenuate chronic stress-induced neurohistochemical abnormalities. Future research however would benefit from an in-depth investigation consisting of a comparative view between their underlying neurochemistry.

As it goes beyond the scope of this study to elaborate upon these latter issues, we acknowledge their relevance within this research. Additional studies are currently being performed to highlight these aspects.

Conclusions

In the present chapter, we illustrate how different antidepressants, characterized by selective and sometimes antagonistic pharmacological profiles, carry out their neurochemical effects by targeting similar intracellular substrates albeit through different mechanisms. Our findings suggest that a critical step in citalopram's mode of action may be constituted by the attenuation of stress-induced functional cortical and subcortical abnormalities. Although not effective in reversing stress-induced phospho-CREB expression changes, long-term SSRI administration did demonstrate positive effects in correcting footshock-induced FOS-ir changes and preventing HPA axis hyperactivity in female rats. The reduction of stress-induced PVN activation and the prevention of hippocampal abnormalities may represent a central step in tianeptine's therapeutic action. These effects may account for tianeptine's ability to prevent the development of HPA axis hyperactivity and avoid persistent exposure to elevated glucocorticoid concentrations. By strongly enhancing CREB phosphorylation, reboxetine may reverse chronic stress-induced reduction of neuronal plasticity, thereby promoting brain structural flexibility and rapid adaptive changes of internal homeostasis in response to prolonged stress exposure. It is of interest to note that all three antidepressant prevented the development of abnormal HPA axis activity.

The maladaptive consequences of stress on neuronal integrity render it one of the primary pathological factors involved in the etiology of stress-related psychiatric disorders. Its detrimental influences on neuronal functioning however, may also account in part for the limited therapeutic power of antidepressants. Given the complex nature of the association between stress, psychiatric disorders, and the mode of action of antidepressants, elucidation of these interactions is of great relevance yet unlikely to be unraveled in the near future. Considering the limited efficacy of today's antidepressants, a full understanding of the mechanisms underlying the contribution of stress on psychopathology, is essential for the development of novel, more successful treatments. This however, shall not prove an easy task, due primarily to our poor comprehension of the mechanisms involved in the development of these psychiatric illnesses and the lack of a clear understanding of the neurobiological substrates mediating the therapeutic effects of antidepressants.

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